Comparative biosynthesis, covalent post-translational modifications and efficiency of prosegment cleavage of the prohormone convertases PC1 and PC2: glycosylation, sulphation and identification of the intracellular site of prosegment cleavage of PC1 and PC2

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We present herein the pulse–chase analysis of the biosynthesis of the prohormone convertases PC1 and PC2 in the endocrine GH_4C_1 cells infected with vaccinia virus recombinants expressing these convertases. Characterization of the pulse-labelled enzymes demonstrated that pro-PC1 (88 kDa) is cleaved into PC1 (83 kDa) and pro-PC2 (75 kDa) into PC2 (68 kDa). Secretion of glycosylated and sulphated PC1 (84 kDa) occurs about 30 min after the onset of biosynthesis, whereas glycosylated and sulphated PC2 (68 kDa) is detected in the medium after between 1 and 2 h. Furthermore, in the case of pro-PC2 only, we observed that a fraction of this precursor escapes glycosylation. A small proportion (about 5 %) of the intracellular glycosylated pro-PC2 (75 kDa) is sulphated, and it is this glycosylated and sulphated precursor that is cleaved into the secretable 68 kDa form of PC2. Major differences in the carbohydrate structures of PC1 and PC2 are demonstrated by the resistance of the secreted PC1 to endoglycosidase H digestion and sensitivity of the secreted PC2 to this enzyme. Inhibition of *N*-glycosylation with tunicamycin caused a dramatic intracellular degradation of these convertases within the endoplasmic reticulum, with the net effect of a reduction in the available activity of PC1 and PC2. These results emphasize the importance of *N*-glycosylation in the folding and stability of PC1 and PC2. Pulse-labelling experiments in uninfected mouse βTC3 and rat Rin m5F insulinoma cells, which endogenously synthesize PC2, showed that, as in infected GH_4C_1 cells, pro-PC2 predominates intracellularly. In order to define the site of prosegment cleavage, pulse–chase analysis was performed at low temperature (15 °C) or after treatment of GH_4C_1 cells with either brefeldin A or carbonyl cyanide *m*-chlorophenylhydrazone. These results demonstrated that the onset of the conversions of pro-PC1 into PC1 and non-glycosylated pro-PC2 into PC2 (65 kDa) occur in a pre-Golgi compartment, presumably within the endoplasmic reticulum. In contrast, pulse labelling in the presence of $Na_2{}^{35}SO_4$ demonstrated that the processing of glycosylated and sulphated pro-PC2 occurs within the Golgi apparatus. In order to test the possibility that zymogen processing is performed by furin, we co-expressed this convertase with either pro-PC1 or pro-PC2. The data demonstrated the inability of furin to cleave either proenzyme.

INTRODUCTION

Cellular communication is accompanied by the release of bioactive peptides and proteins which act as the messengers or carriers of information. These active moieties are usually derived from inactive larger precursor proteins that undergo a number of post-translational modifications. Precursor processing at either pairs of or single basic residues represents one of the early post-translational events [1–3]. Recently, the search for the proteinase(s) responsible for the cleavage of proproteins at pairs of basic residues culminated in the identification of six different gene products, representing mammalian kexin/subtilisin-like serine proteinases [1,4]. These proprotein convertases were called PC1 [5] (also called PC3 in [6]), PC2 [7,8], furin [9], PACE4 [10], PC4 [11,12] and PC5 [4,13]. Analysis of the gene regulation [14,15] and tissue distribution of some of these convertases demonstrated a unique pattern for each enzyme. Thus, whereas PC1 and PC2 are only found in endocrine and neuroendocrine tissues and cells [4–8,16], furin [17,18] and PACE4 [10] exhibit a ubiquitous tissue distribution and PC5 seems to localize only within a subset of endocrine and non-endocrine cells. In contrast, PC4 is expressed primarily within testicular germ cells, including round spermatids [11,12] and pachytene spermatocytes [12]. The unique but widespread tissue distribution of the mRNA transcripts of individual mammalian kexin/subtilisin-like convertases suggests that each proteinase, either alone or in combination with the others, and in a tissue-specific manner, could potentially activate a variety of proproteins and prohormones [1,4]. In this context, furin was reported to cleave efficiently precursors that are usually expressed in cells devoid of secretory granules, suggesting that this convertase is mostly involved in the processing of proproteins negotiating the constitutive pathway of secretion [19,20]. In contrast, PC1 and PC2 have been shown to represent major processing enzymes of prohormones destined to the regulated secretory pathway [21–23], in agreement with the observed expression of these convertases primarily within endocrine and neuroendocrine tissues and cells [4–8,14,16,21].

The alignment of the sequences of PC1, PC2, furin, PACE4, PC4 and PC5 with that of subtilisin suggested that each convertase possesses a signal peptide followed by a unique prosegment of about 83–90 amino acids. By analogy with prosubtilisin [24], this prosegment might have to be excised in

Abbreviations used: ER, endoplasmic reticulum; m, mouse; h, human; p, porcine; POMC, pro-opiomelanocortin; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Endo H, endoglycosidase H.

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order to generate an active proteinase [1,4–8,10–12,23,25,26]. Recent studies with the convertases kexin [27,28] and furin [29] demonstrated that these two processing enzymes are able to autocatalytically excise their prosegments. In the case of the yeast kexin [27,28], this reaction seems to take place within the endoplasmic reticulum (ER), resulting in an active enzyme [30]. In contrast, the ER may not represent a favourable milieu for the efficient autoactivation of furin [31].

As the rate of removal of the N-terminal prosegment could conceivably represent a mechanism by which the cell could control the efficiency of proprotein processing [23], we undertook the comparative analysis of the prosegment cleavage of PC1 and PC2 in GH_4C_1 cells. The cDNA structures of PC1 [5,6] and PC2 [7,8] predict three potential *N*-glycosylation sites for each enzyme. Accordingly, we also investigated the potential role of glycosylation in the processing, secretion and activity of these convertases. Our data permit us to define the ER and the *trans*-Golgi network as the intracellular sites of pro-PC1 and glycosylated pro-PC2 processing respectively. The mechanism of prosegment cleavage of PC1 and PC2 is not yet known, nor is it defined to be an autocatalytic or heterocatalytic process. Therefore we also investigated the possibility that the ubiquitous convertase furin [17] participates in this zymogen-activation reaction.

MATERIALS AND METHODS

Vaccinia virus recombinants of mouse (m)PC1, human (h)PC1, mPC2, hfurin, mouse pro-opiomelanocortin (mPOMC) and porcine (p)POMC

The purified recombinant vaccinia viruses of mPC1 (VV:mPC1) [5] and mouse PC2 (VV:mPC2) [8] were the same as those previously reported [21]. The vaccinia virus recombinant of hPC1 (VVtm1:hPC1) was prepared from the cDNA of hPC1 [32] using the pTM-1 transfer vector [33]. The recombinant VV:hfurin was prepared using the cDNA of human furin [9] (a gift from Dr. A. Rehemtulla, Genetics Institute, Boston, MA, U.S.A.) subcloned in the pVV3 transfer vector as reported for VV:mPC1 and VV:mPC2 [21]. The vaccinia virus recombinant of mPOMC (VV:mPOMC) was a gift from Dr. G. Thomas (Vollum Institute, Portland, OR, U.S.A.) and that of pPOMC (VV:pPOMC) was obtained as described [34].

Cellular infections

In this work we have used three types of cells plated on 60 cm^2 dishes: a GH_4C_1 rat somatomammotroph cell line, a βTC3 mouse pancreatic and a Rin m5F rat insulinoma cell line. The vaccinia virus used for infection of all the cells consisted of either the wild-type virus (VV:WT) or the recombinant viruses, and the infections were performed at a multiplicity of infection of 1 plaque-forming unit (p.f.u.)/cell, except for the VVtm1:hPC1 where we performed an additional co-infection with 1 p.f.u./cell of VTF7-3 vaccinia virus recombinant which expresses the bacteriophage T7 RNA polymerase [33].

Metabolic labelling of cells

After the infection of GH_4C_1 cells with VV:PC1 and VV:PC2, we pulsed the cells (in duplicate and sometimes in quadruplicate) in the presence of 1 mCi of either L-[^{35}S]methionine, L-[^{3}H]phenylalanine, L-[^{3}H]leucine or $Na_2{}^{35}SO_4$. The radiolabelled products were either analysed directly or the L-[^{35}S]methionine pulse was followed by chase periods in the presence of 10 mM methionine.

At the end of the pulse or pulse–chase periods, the medium was removed, centrifuged for 10 min at 300 *g* after the addition of protease inhibitors including: phenylmethanesulphonyl fluoride (100 μg/ml), aprotinin (2 μg/ml) and EDTA (1 mM). The cells were washed once in PBS-M (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 4.2 mM Na_2HPO_4 and 1 mM $MgCl_2$), and then lysed in a Nonidet P40 lysis buffer containing 50 mM Tris, 150 mM NaCl, 0.05% SDS and 1% Nonidet P40 and the same protease inhibitors as above. The cells were freeze–thawed three times, sonicated for 30 s, centrifuged and the supernatant was immunoprecipitated with either PC1- or PC2-specific antibodies.

In experiments with brefeldin A or tunicamycin, the cells were preincubated for either 60 min or 180 min with 5 μg/ml either brefeldin A (Epicentre) or tunicamycin (Sigma), and then pulse and pulse–chase analysis were performed. In experiments with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), the cells were first pulse-labelled and then chased in the presence or absence of 40 μM CCCP (Sigma). In temperature block experiments, the cells were labelled at 37 °C and then chased at either 15 °C or 37 °C.

Analysis of radiolabelled PC1 and PC2

In order to study the secreted and cellular forms of PC1 and PC2 radiolabelled with [^{35}S]methionine, the media or cell extracts were immunoprecipitated with either an mPC1 (AbC-mPC1) or an mPC2 (AbC-mPC2) polyclonal antibody directed against the C-terminal segment 629–726 of mPC1 [5] or 529–637 of mPC2 [8] respectively as described [23]. For displacement purposes, in some cases before immunoprecipitation, we preincubated the antibodies for about 1 h with 50 μg of the segments comprising residues 629–726 of mPC1 or 529–637 of mPC2, obtained by thrombin digestion of bacterially produced fusion proteins to glutathione S-transferase [23]. We also analysed the labelled PC1-related proteins by immunoprecipitation with a polyclonal antibody AbN-mPC1, directed against the N-terminal segment 84–100 of mPC1 [5], which was kindly provided by Dr. Iris Lindberg (Louisiana State University, New Orleans, LA, U.S.A.). Immunoprecipitated radiolabelled proteins were resolved by SDS/PAGE on an 8% acrylamide gel. The low-range prestained SDS/PAGE molecular-mass standards used were obtained from Bio-Rad. For analytical purposes, the gels were treated with Entensify as specified by the manufacturer (DuPont), dried and autoradiographed on a Kodak XAR-5 film. For preparative purposes, identical gels were sliced (1 mm), and the radiolabelled proteins were electroeluted and subjected to microsequence analysis on an Applied Biosystems model 470A sequenator.

Digestion with endoglycosidase H (Endo H), *N*-glycosidase F and arylsulphatase

Immunoprecipitates (15 μl) of media and cell extracts (dissolved in Laemmli buffer) obtained from pulse and pulse–chased GH_4C_1 cells infected with either VV:mPC1 or VV:mPC2 were digested with either 4.6 or 23 munits of Endo H or peptide *N*-glycosidase F (4 units), as described by the manufacturer (Oxford GlycoSystems), or 1 units of arylsulphatase (Sigma) for 10 h and/or 24 h at 37 °C. The radiolabelled digestion products were resolved by SDS/PAGE as described above.

RESULTS

Recently we demonstrated that pro-PC1 and pro-PC2 are cleaved intracellularly at pairs of basic residues resulting in the excision of their prosegments. Furthermore, we also showed that these

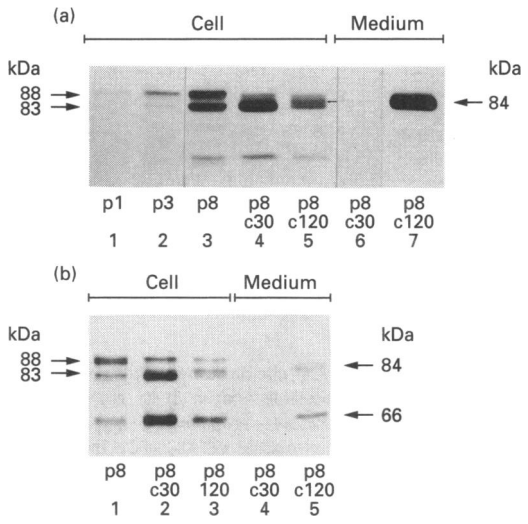

Figure 1 Pulse–chase analysis of the biosynthesis of mPC1 in GH_4C_1 cells

GH_4C_1 cells infected with VV:mPC1 were pulse-labelled (p) with [^{35}S]methionine for 1 (p1), 3 (p3) or 8 (p8) min. The 8 min pulse (p8) was followed by a chase (c) with unlabelled methionine for 30 min (p8/c30) or 120 min (p8/c120). The total of the cell extracts and medium were immunoprecipitated with (**a**) a C-terminal mPC1-specific antibody AbC-mPC1 or (**b**) an N-terminal mPC1-specific antiserum. The immunoprecipitates were separated by SDS/PAGE and fluorographed as described [23]. The resulting autoradiogram also depicts the estimated molecular mass of the intracellular and secreted forms of PC1, based on the migration position of the molecular-mass standards obtained from Bio-Rad.

two proenzymes are processed to different extents independently of whether the cell line contains dense core secretory granules. We also showed that cells mostly secrete the N-terminal-cleaved forms of PC1 and PC2, and that intracellularly the pro-form of PC2 predominates [23]. However, these static 6 h pulse experiments did not measure the processing rate nor did they identify the cellular compartment in which the prosegment removal of each convertase occurs. In the present study we used a pulse–chase protocol to examine the comparative rates of removal of the prosegment of PC1 and PC2 in GH_4C_1 cells.

Pulse–chase analysis of the biosynthesis of PC1 in GH_4C_1 cells

The data in Figure 1(a) show the presence of three PC1-related proteins immunoprecipitable with the C-terminal antibody (AbC-mPC1). These proteins migrate with apparent molecular masses of 88, 84 and 83 kDa. We recently reported the microsequence identification of the 88 kDa protein as pro-PC1 [35] (originally reported to migrate at an estimated molecular mass of 87 kDa) and the 83, 84 kDa proteins as PC1 [23] (originally reported to migrate at an estimated molecular mass of 80 kDa). The results show that the excision of the prosegment of pro-PC1 (88 kDa) is quite fast, as PC1 (83 kDa) appears after only 1, 3 or 8 min of pulse-labelling (Figure 1a, lanes 1–3), and the excision is almost complete by 30 min of chase (Figure 1a, lane 4). In another similar experiment, in which the cells were pulse-labelled for 5 min and chased for 20 min, we observed the same extent of transformation of pro-PC1 to PC1 (not shown). These data suggest an early and efficient removal of the 83-amino acid prosegment of pro-PC1 during its biosynthesis.

We also notice that an 84 kDa protein intermediate in size between pro-PC1 (88 kDa) and PC1 (83 kDa) is apparent intracellularly at 120 min of chase (Figure 1a, lane 5; small arrow). Furthermore, PC1 can be detected in the medium after 30 min of chase. This secreted form of PC1 migrates with an apparent molecular mass of 84 kDa, which is identical with that estimated for the intermediate form (Figure 1a, lanes 6 and 7). The data from Figure 1(a) also show that most of the radiolabelled PC1 is secreted from the cell by 120 min of chase (Figure 1a, lane 7). Although in Figure 1(a) we observed an intracellular PC1-related protein migrating with an apparent molecular size of 66 kDa (possibly representing an N-terminally truncated PC1), this minor form is not secreted into the medium.

Using an N-terminally directed mPC1 antibody (AbN-mPC1), which recognizes the first 17 amino acids of PC1 (residues 84–100), Vindrolla and Lindberg [36] reported that, in AtT-20 cells, PC1 is further slowly cleaved at its C-terminus, resulting in a 66 kDa form. Using the same antiserum, we also immunoprecipitated the supernatant of the AbC-mPC1 precipitation presented above (Figure 1a). Accordingly, we also detected a 66 kDa form appearing intracellularly in the 8 min pulse, and, like the 83 kDa form, its level peaks at 30 min (Figure 1b, lane 2). GH_4C_1 cells were also pulsed with [^{35}S]methionine for 15 min and then chased for 3 h, and the medium was immunoprecipitated with AbN-mPC1. The results of the SDS/PAGE of this immunoprecipitate allowed the elution of about 8000 c.p.m. of the labelled 66 kDa form of PC1 (not shown). The microsequence of this material demonstrates the presence of a methionine at sequence position 15 (not shown). From the deduced protein sequence of mPC1 [5], we conclude that the 83–84 kDa [22] and the 66 kDa forms of PC1 have the same N-termini. As the secreted 66 kDa form is not recognized by our C-terminal antibody, these data agree with the observation of Vindrolla and Lindberg [36] who suggested that it represents PC1 truncated at its C-terminus, at an as yet unspecified site. It is important to note that the relative amounts of the various forms of PC1 shown in Figure 1(b) are not representative of their actual ratio, since the pro-PC1 and PC1 forms seen are remnants of the immunoprecipitation with the C-terminal antibody.

Pulse-labelling experiments in the presence of $Na_2{}^{35}SO_4$ demonstrate that only the 84 kDa form of PC1 is sulphated both intracellularly and in the medium. Although it decreased the size of PC1 to about 83 kDa, digestion with endoglycosidase F did not diminish the intensity of the label (not shown), suggesting the absence of sulphation on an *N*-glycosyl moeity. Pro-PC1 (88 kDa), the 83 kDa form of PC1 and the secreted 66 kDa form recognized by the N-terminal antiserum were not labelled with [^{35}S]sulphate (not shown). We believe that sulphation occurs at a specific tyrosine residue, as digestion of the sulphated PC1 (84 kDa) with arylsulphatase eliminated all the label on this protein (not shown), which also argues for the absence of sulphation on an *O*-glycosylation site. Furthermore, the lack of sulphation in the 66 kDa form suggests that the sulphation site is within the segment 629–726 of mPC1.

Pulse–chase analysis of the biosynthesis of PC2 in GH_4C_1 cells

The data from Figure 2(a) demonstrate, at pulse times as short as 3 or 10 min, the intracellular presence of three PC2-immunoreactive proteins with apparent molecular masses of 75, 70 and 65 kDa. The microsequence of the 75 kDa form indicates that it represents pro-PC2 [23,35]. The low levels of the 70 kDa form do not allow an unambiguous microsequence identification of either the [^{35}S]methionine- or [^{3}H]leucine-labelled product. The microsequence of the 65 kDa form labelled with [^{35}S]methionine reveals the presence of Met-12 and Met-16, possibly because it is the sequence of a mixture of two PC2-related proteins obtained by the cleavage of pro-mPC2 at either

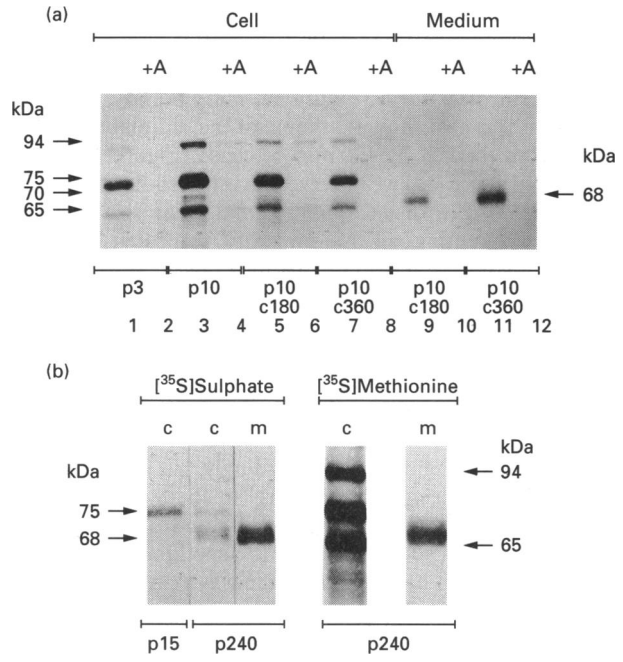

Figure 2 Pulse–chase analysis of the biosynthesis of mPC2 in GH_4C_1 cells

GH_4C_1 cells infected with VV:mPC2 were pulse-labelled with [^{35}S]methionine for either 3 (p3) or 10 (p10) min (**a**). The 10 min pulse (p10) was followed by a chase with unlabelled methionine for 180 min (p10/c180) or 360 min (p10/c360). (**b**) The cells were pulse-labelled with [^{35}S]sulphate for 15 min and 240 min, or [^{35}S]methionine for 240 min. The total of the cell (c) extracts and medium (m) were immunoprecipitated with AbC-mPC2 in the presence (+A) and/or absence of 50 μg of a competing antigen representing the segment 529–637 of mPC2 [8]. The SDS/PAGE analysis and autoradiography conditions were identical with those in Figure 1.

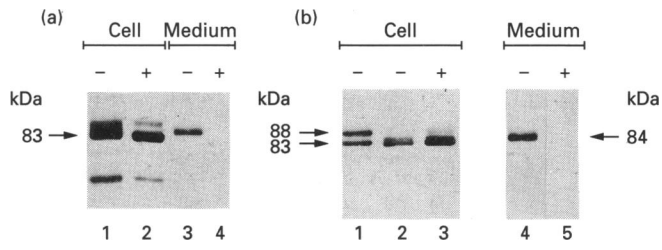

Figure 3 Effects of brefeldin A and CCCP on the prosegment cleavage of PC1 in GH_4C_1 cells

GH_4C_1 cells infected with VV:mPC1 were pulse-labelled with [^{35}S]methionine for 120 min in the absence (−) or presence (+) of brefeldin A (**a**). In (**b**) the cells were pulsed for 5 min and then chased for 120 min in the absence (−) or presence (+) of CCCP. The total of the cell extracts and medium were immunoprecipitated with AbC-mPC1. The SDS/PAGE analysis and autoradiography conditions were identical with those in Figure 1.

Lys-Arg-Arg-Arg^{84}↓ or Lys-Arg-Arg-Arg^{56}↓ respectively [8]. However, we also notice that, unlike the PC2 form secreted into the medium [23], the sequencing yield of these PC2-related intracellular proteins is very low.

Analysis of the intracellular forms obtained after 3 h or 6 h of chase (Figure 2a, lanes 5 and 7) demonstrate a gradual decrease in the level of the pro-PC2 (75 kDa). We also see a parallel gradual decrease in the 65 kDa form and the disappearance of the intermediate 70 kDa form. However, no increase in the level of either the 65 or the 70 kDa forms is observed at any time during the chase. A mature 68 kDa form is primarily detected in the medium [23] (originally reported to migrate at an apparent molecular mass of 65 kDa), the level of which increases with chase time from 3 to 6 h (Figure 2a, lanes 9 and 11). It should be noted that [^{35}S]methionine-labelled PC2 is detectable in the medium only after 2 h of chase (not shown). The microsequence of the 68 kDa form in the medium shows that it represents a PC2 form in which the N-terminal 84-amino-acid segment of pro-PC2 has been removed via cleavage at the predicted second tetrabasic sequence, i.e. after Lys-Arg-Arg-Arg^{84}↓ [8,23]. In the GH_4C_1 cell line used in this study, the intracellular 75 kDa pro-PC2 form is still detectable even up to 22 h of chase time (not shown).

As shown in Figure 2(b), comparison of cells pulse-labelled with $Na_2{}^{35}SO_4$ or [^{35}S]methionine for 4 h shows that a small fraction (about 5%) of pro-PC2 (75 kDa) is sulphated intracellularly, whereas in the medium about 80% of the 68 kDa PC2 is sulphated. Furthermore, the 15 min pulse data emphasize that sulphation of pro-PC2 precedes its processing. We also note that the 65, 70 and 94 kDa immunoprecipitable PC2-containing proteins (Figure 2a) are not sulphated. As the 94 kDa protein is larger than the size of pro-PC2, we have not yet defined its molecular composition. Although in cell extracts we could barely detect the 68 kDa form of the [^{35}S]methionine-labelled PC2 (Figure 2a), the ratio of [^{35}S]sulphate-labelled PC2 (68 kDa) to pro-PC2 (75 kDa) is about 2:1 (Figure 2b). This suggests that the small portion of sulphated pro-PC2 detected in the cell at any given time is converted into the secreted PC2 (68 kDa).

Digestion of the sulphated pro-PC2 and PC2 with Endo H or *N*-glycosidase F, although clearly decreasing the size of these proteins, did not diminish the intensity of the label (not shown). Accordingly, like PC1, sulphation of PC2 does not occur on the carbohydrate chains attached to asparagine residues, but rather occurs on a tyrosine residue, as arylsulphatase digestion eliminated all the sulphate label on PC2.

Identification of the sites of cleavage of the prosegments of PC1 and PC2

From the data of Figure 1, the processing of pro-PC1 to PC1 was shown to be both efficient and rapid, suggesting that such a cleavage may occur within the ER and continue in the *cis*-Golgi compartment. In order to define more precisely the site at which the onset of such a processing step occurs, we used three different treatments that disrupt the transport of proteins from the ER to the Golgi complex. In the first two, we inhibited the migration of pulse-labelled proteins to the Golgi apparatus by chasing the cells either at 15 °C [37] or in the presence of CCCP [38], which depletes the cellular ATP levels and hence blocks the movement of proteins from the ER to the Golgi complex. In the latter treatment we pretreated the cells with the drug brefeldin A, a unique fungal metabolite known to cause disassembly of the Golgi complex and accumulation of secretory proteins in the ER [39].

Treatments with either brefeldin A (Figure 3a) or CCCP (Figure 3b) did not affect the extent of processing of pro-PC1 to PC1, as judged by both N-terminal (not shown) and C-terminal immunoprecipitations. Furthermore, as expected, both drugs blocked the basal secretion of PC1 into the medium. Interestingly, the intermediate-sized PC1 (84 kDa) only appears in the absence of brefeldin A preincubation (Figure 3a, lane 1). This is consistent with the identification of this 84 kDa form as the trimmed and remodelled PC1 protein which has already reached the *medial* Golgi cisternae.

Although it blocked the basal secretion of PC2, treatment of the GH_4C_1 cells with brefeldin A (Figure 4a) or CCCP (Figure

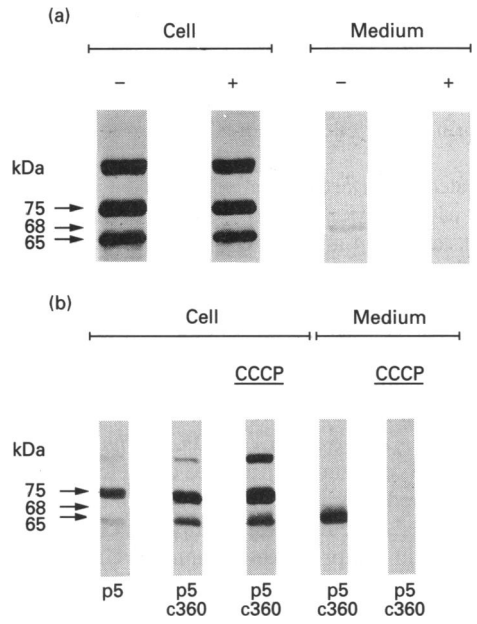

Figure 4 Effects of brefeldin A and CCCP on the prosegment cleavage of PC2 in GH_4C_1 cells

GH_4C_1 cells infected with VV:mPC2 were pulse-labelled with [^{35}S]methionine for 240 min in the absence (−) or presence (+) of brefeldin A (**a**). In (**b**) the cells were pulsed for 5 min and then chased for 360 min in the absence or presence of CCCP. The totals of the cell extracts and medium were immunoprecipitated with AbC-mPC2. The SDS/PAGE analysis and autoradiography conditions were identical with those in Figure 1.

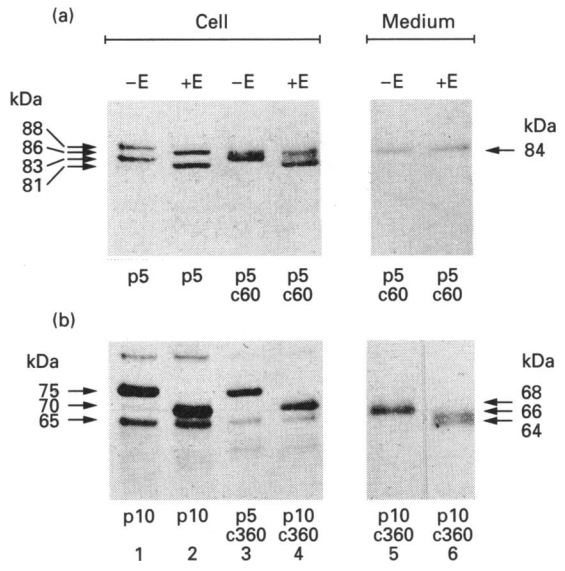

Figure 5 Endo H digestions

GH_4C_1 cells were infected with (**a**) VV:mPC1 and then pulse-labelled with [^{35}S]methionine for 5 min and then chased for 60 min or (**b**) VV:mPC2, pulse-labelled with [^{35}S]methionine for 10 min and then chased for 360 min. The total of the cell extracts and medium were immunoprecipitated with the C-terminal (AbC-mPC1 or AbC-mPC2) antisera. Then 10% of these immunoprecipitates were incubated in the absence (−E) or presence (+E) of Endo H for 10 h at 37 °C. The digestion products and controls were then separated by SDS/PAGE and the molecular masses were estimated as described in the legend of Figure 1.

4b) did not affect the inefficient conversion of pro-PC2 to PC2, as demonstrated by the relative abundance of pro-PC2 (75 kDa). Finally, we also compared data obtained from pulse–chase analysis performed at either 37 °C or 15 °C. The results revealed that, although the basal release of PC1 and PC2 is effectively blocked at 15 °C, the conversions of pro-PC1 and pro-PC2 into PC1 and PC2 respectively are not affected by this treatment (not shown). The conclusions drawn from these data agree with those obtained from the brefeldin A and CCCP treatments, and all of them strongly suggest that the cleavage of the prosegment of pro-PC1 and non-glycosylated pro-PC2 starts in a pre-Golgi compartment, likely to be within the ER itself.

Endo H digestion of the different PC1 and PC2 forms

As shown in Figure 5(a) (lanes 1 and 2), pro-PC1 (88 kDa) and PC1 (83 kDa), obtained after a pulse of 5 min, are both sensitive to Endo H treatment. However, the 84 kDa form of PC1, which is released into the medium after a 60 min chase, is resistant to Endo H digestion (Figure 5a, lanes 5 and 6). We note that even after 1 h of chase, a significant amount of Endo H-sensitive PC1 (83 kDa) is still detectable intracellularly (Figure 5a, lanes 3 and 4).

The results of Figure 5(b) demonstrate that intracellularly pro-PC2 (75 kDa) is always sensitive to Endo H digestion, even after 6 h of chase (lanes 1–4). These data suggest (i) that the structure of the oligosaccharide moieties of pro-PC2 are of a high-mannose type (and hence sensitive to Endo H) and/or (ii) that pro-PC2 slowly exits the ER and is then rapidly transported through the Golgi apparatus and then processed and secreted. We believe that both hypotheses are correct, as we can only detect a small portion of the total pro-PC2 in a sulphated form, which is the precursor of the secreted PC2 (Figure 2b). We also note that in the medium of GH_4C_1 cells, PC2 (68 kDa) is digested by Endo H into roughly an equal mixture of 66 and 64 kDa forms (Figure 5b, lanes 5 and 6). This is not due to partial digestion, as increasing both the amount of Endo H (5-fold) and the digestion time (from 10 h to 24 h) did not alter the level of either form (not shown). These data suggest the presence of heterogeneous carbohydrate structures attached to the putative three *N*-glycosylation sites in the secreted form of PC2 [8] and that possibly some of these are occupied by high-mannose-type oligosaccharides, which renders them Endo H-sensitive. Finally, Endo H digestion of the electroeluted non-sulphated 65 and 70 kDa forms observed early during the chase (Figure 2a) also show that both forms are Endo H-resistant, supporting the hypothesis that these are not *N*-glycosylated. Therefore our data suggest that the processing of non-glycosylated pro-PC2 occurs in the ER (Figure 4), whereas the processing of the glycosylated (Endo H-sensitive) and sulphated pro-PC2 occurs within the Golgi apparatus (Figure 2b).

Effect of tunicamycin on the processing, stability, basal secretion and activity of PC1 and PC2

In order to investigate whether *N*-glycosylation affects the processing of pro-PC1 and pro-PC2, and consequently the activity of these convertases, we preincubated the GH_4C_1 cells for 3 h with 5 μg/ml tunicamycin. SDS/PAGE analysis of the [^{35}S]methionine-labelled PC1 and PC2 proteins immunoprecipitated from the cell extracts and media are shown in Figures 6(a) and 6(b). The data confirm that pro-PC1 (Figure 6a, lanes 1 and 2) and pro-PC2 (Figure 6b, lanes 1 and 2) are *N*-

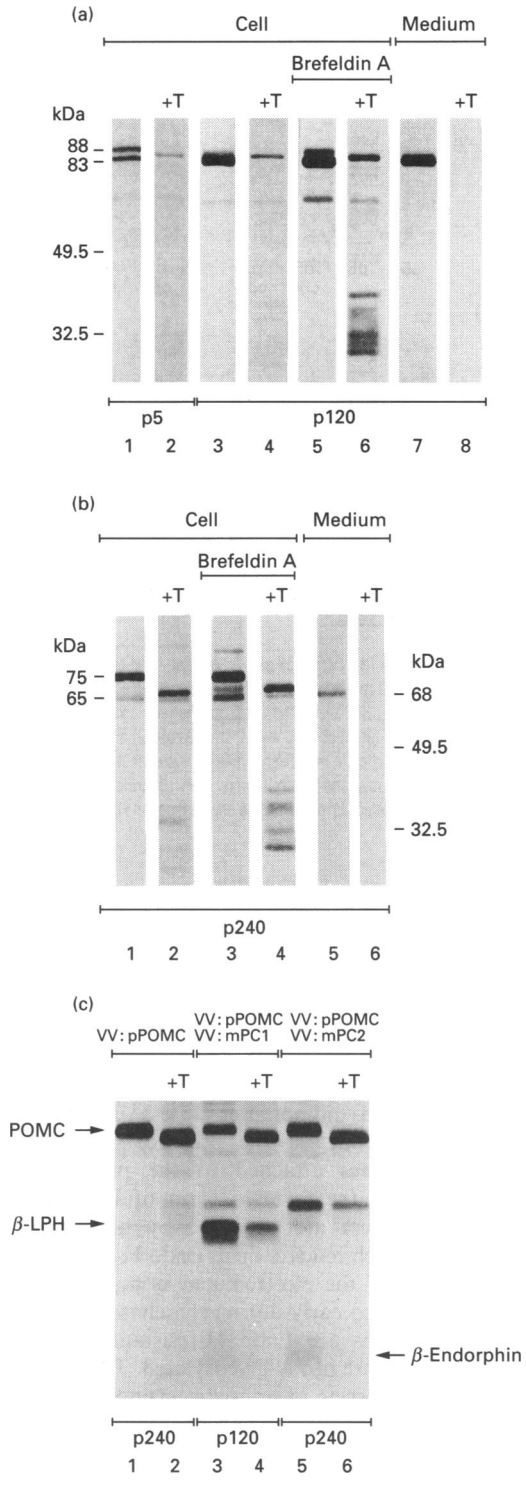

Figure 6 Tunicamycin effects in GH_4C_1 cells

GH_4C_1 cells were infected with (**a**) VV:mPC1 and then pulse-labelled with [^{35}S]methionine for 5 min (lanes 1 and 2) or 120 min (lanes 3–8) or (**b**) VV:mPC2 and then pulse-labelled with [^{35}S]methionine for 240 min or (**c**) VV:pPOMC (lanes 1 and 2) or VV:pPOMC + VV:mPC1 (lanes 3 and 4) or VV:pPOMC + VV:mPC2 (lanes 5 and 6) and then pulse-labelled with [^{35}S]methionine for either 240 min (lanes 1, 2, 5 and 6) or 120 min (lanes 3 and 4). The results of the experiments involving tunicamycin addition (+T) are shown in lanes 2, 4, 6 and 8 of (**a**) and lanes 2, 4 and 6 of (**b**) and (**c**). The effects of the further addition of brefeldin A are shown in lanes 5 and 6 of (**a**) and lanes 3 and 4 of (**b**). The total of the cell extracts (**a** and **b**) and media (**a**, **b**, **c**) were immunoprecipitated with AbC-mPC1 (**a**) or AbC-mPC2 (**b**) or β-endorphin (**c**) antisera. The SDS/PAGE analysis and autoradiography conditions were identical with those in Figure 1. It should be noted that, in (**a**), the results in lanes 1 and 2, lanes 3, 4, 7 and 8 and lanes 5 and 6 are derived from three different SDS/PAGE experiments. Similarly, in (**b**), the results in lanes 1, 2, 5 and 6 and lanes 3 and 4 are obtained from two different gels. Abbreviation: β-LPH, β-lipotropin.

glycosylated, as tunicamycin reduces their molecular masses by approx. 3–5 kDa. We also note that the non-glycosylated pro-PC2 obtained after tunicamycin treatment migrates as a 70 kDa entity (Figure 6b, lanes 2 and 4). These results suggest that the 70 kDa form observed in Figure 2(a) is non-glycosylated pro-PC2.

Unexpectedly, the prevention of *N*-glycosylation of both PC1 and PC2 causes their considerable intracellular degradation (Figure 6a, lanes 2, 4 and 6 and Figure 6b, lanes 2 and 4). This degradation generates multiple polypeptides of lower molecular mass. The consequence of this degradation is a much reduced level of basally secreted PC1 (Figure 6a, lanes 7 and 8) and PC2 (Figure 6b, lanes 5 and 6). In order to define the organelle in which such degradation occurs, we have also incubated the cells with both tunicamycin and brefeldin A. We observed that both PC1 (Figure 6a, lanes 5 and 6) and PC2 (Figure 6b, lanes 3 and 4) are degraded, even in the presence of brefeldin A, suggesting that the observed degradation occurs within the ER. Furthermore, the data also demonstrate that pro-PC1 and pro-PC2 are still detectable in the presence of tunicamycin and brefeldin A, suggesting that these proforms are degraded to a much lesser extent than either PC1 or PC2.

As earlier experiments [40] demonstrated that tunicamycin does not affect the secretion or cause the degradation of pituitary POMC, we investigated whether the removal of the *N*-glycosylation of PC1 and PC2 can also affect their proven ability to process POMC [21]. As shown in Figure 6(c), tunicamycin also reduces the molecular mass of POMC by about 3 kDa (lanes 1 and 2). We also observed that the ability to process POMC by either PC1 into β-lipotropin (Figure 6c, lanes 3 and 4) or PC2 into a high-molecular-mass intermediate and β-endorphin (lanes 5 and 6) is clearly diminished (lanes 4 and 6). The above data suggest that, although much of the PC1 and PC2 synthesized *de novo* is degraded in the absence of *N*-glycosylation, there is some residual activity, which enables the processing of POMC. It is also possible that this activity is due in part to the presence of residual glycosylated enzymes synthesized during the overnight infection and before the addition of tunicamycin (see the Materials and methods section).

The low level of pro-PC2 processing is due to an intrinsic property of this convertase

In order to examine whether the conversion of pro-PC2 into PC2 observed in GH_4C_1 cells infected with VV:mPC2 is somehow slowed down by the vaccinia infection, we investigated the intracellular processing of endogenous PC2 in two cell types known to contain important amounts of PC2. These included the mouse βTC3 and rat Rin m5F insulinoma cells which efficiently process proinsulin to insulin and which are rich in PC2 mRNA ([5,8,14]; R. Day, unpublished work).

The two cell types used were pulse-labelled with [^{35}S]methionine for 10 min and chased for 3 h in the absence of infection or after vaccinia virus infection with VV:mPC2. The results of SDS/PAGE analysis of the immunoprecipitation of PC2 are shown in Figure 7. In the absence of vaccinia virus infection, we calculate that 53% and 47% of the PC2-related proteins are found as pro-PC2 in βTC3 and Rin m5F cells respectively (lanes 1 and 3). Although not shown, after a 60 min [^{35}S]methionine pulse and a 360 min chase of βTC3 cells, the level of intracellular pro-PC2 decreased by about 90%, with little

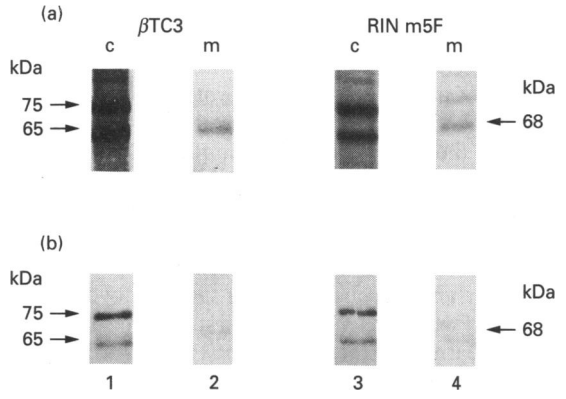

Figure 7 Cell type and pro-PC2 processing

Non-infected (**a**) rat Rin m5F or mouse βTC3 cells from 60 cm^2 dishes or VV:mPC2-infected cells (**b**), in this sole instance, from 10 cm^2 dishes were pulse-labelled with [^{35}S]methionine for 10 min and then chased for 180 min. The totals of the cell extracts (c) and medium (m) were immunoprecipitated with AbC-mPC2. The SDS/PAGE analysis and autoradiography conditions were identical with those in Figure 1.

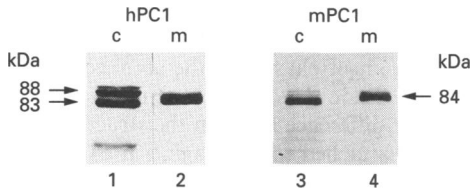

Figure 8 Pulse-labelling analysis of VVtm1:hPC1- and VV:mPC1-infected GH_4C_1 cells

GH_4C_1 cells that were co-infected with either VVtm1:hPC1 and VTF7-3 at 1 p.f.u./cell of each virus (lanes 1 and 2) or mouse VV:mPC1 at 1 p.f.u./cell (lanes 3 and 4) were pulse-labelled with [^{35}S]methionine for 120 min. The totals of the cell extracts (c) and medium (m) were immunoprecipitated with AbC-mPC1. The SDS/PAGE analysis and autoradiography conditions were identical with those in Figure 1.

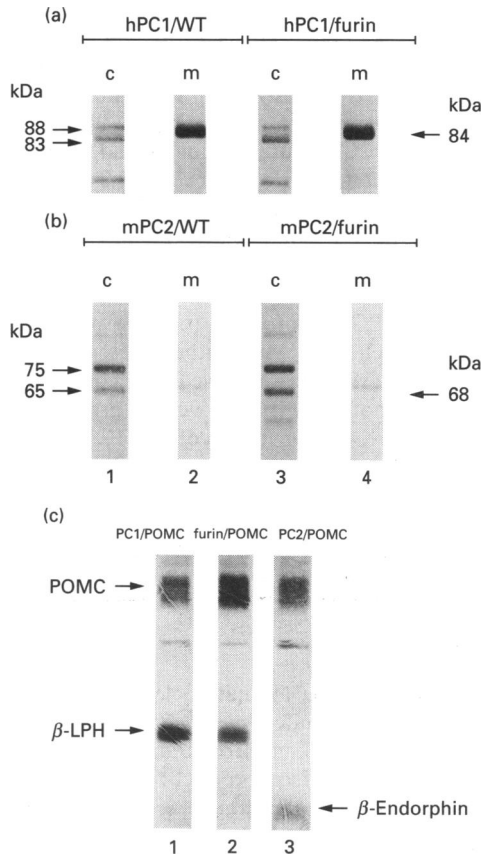

Figure 9 Effect of furin on the processing of pro-PC1 and pro-PC2

GH_4C_1 cells were infected with (**a**) VVtm1:hPC1 + VTF7-3 + VV:WT or VVtm1:hPC1 + VTF7-3 + VV:hfurin, (**b**) VV:mPC2 + VV:WT or VV:mPC2 + VV:hfurin or (**c**) VV:mPC1 + VV:mPOMC or VV:hfurin + VV:mPOMC or VV:mPC2 + VV:mPOMC. These cells were then pulse-labelled with [^{35}S]methionine for either 10 min and then chased for 180 min (**a**, **b**) or for 120 min (**c**). The cell extracts (c) and/or media (m) were then immunoprecipitated with (**a**) AbC-mPC1, (**b**) AbC-mPC2 or (**c**) a β-endorphin-specific antiserum [20]. The SDS/PAGE analysis and autoradiography conditions were identical with those in Figure 1. Abbreviation: β-LPH, β-lipotropin.

change in the amount of the 65 kDa form. These data suggest that the processed PC2 is preferentially released from the cell and does not accumulate intracellularly.

As shown in Figure 7, when either βTC3 or Rin m5F cells were infected with VV:mPC2 before the pulse–chase labelling, there was a slight increase in the level of pro-PC2 (56% of the total PC2-immunoprecipitable proteins in both cells), suggesting that cellular infection has a minor effect on the observed extent of cleavage of the prosegment of PC2 (lanes 2 and 4). These data demonstrate that the observed limited processing of pro-PC2 is not likely to be an artifact or to be caused by the vaccinia virus infection and probably depends on some intrinsic characteristic of this precursor. The results agree with those recently reported by Guest et al. [41], who showed that in pancreatic islets of Langerhans processing of pro-PC2 seems to proceed to completion within 7 h of the chase.

Furin does not cleave the N-terminal prosegment of pro-PC1 and pro-PC2

The results of Figure 1 demonstrate that, using mouse PC1 recombinant VV:mPC1, this convertase is transformed from the 88 kDa to the 83 kDa form within the first few minutes of synthesis. As shown in Figure 8, when GH_4C_1 cells were co-infected with VVtm1:hPC1 and VTF7-3 and pulsed for 1 h with either [^{35}S]methionine or [^{3}H]phenylalanine, there was a 3-fold increase in the level of secreted hPC1 (84 kDa) compared with a similar VV:mPC1 infection (compare lanes 2 and 4). Surprisingly, we also detected much more important amounts of the intracellular pro-hPC1 (88 kDa) as compared with pro-mPC1 (Figure 8, lanes 1 and 3). As expected from the cDNA sequence of hPC1 [32], the microsequence identification of pro-hPC1 (88 kDa) and hPC1 (83 kDa) revealed a Phe-4 and Phe-11 respectively. Therefore, following the predicted signal peptidase cleavage site, pro-hPC1 is cleaved intracellularly at the same Arg-Ser-Lys-Arg↓ position as pro-mPC1 [23], to produce hPC1 which represents residues 84–726 of pro-hPC1 [32].

Using the hPC1 and mPC2 vaccinia virus recombinants, we investigated the possible participation of furin in the intracellular cleavage of the N-terminal prosegments of PC1 and/or PC2. GH_4C_1 cells were co-infected with either VVtm1:hPC1, VTF7-3 and VV:hfurin or VVtm1:hPC1, VTF7-3 and the wild-type control VV:WT. Similarly, for PC2 we co-infected these cells with VV:mPC2 and VV:hfurin or with VV:mPC2 and VV:WT.

The SDS/PAGE analysis of the immunoprecipitation of the [^{35}S]methionine 10 min pulse-labelled and 3 h-chased GH_4C_1 cell extracts and media is shown in Figure 9. The data show that, intracellularly, the co-expression of furin does not alter the processing of the pro-PC1 into PC1 (Figure 9a, lanes 1 and 3) and pro-PC2 into PC2 (Figure 9b lanes 1 and 3). We also note that the levels of secreted PC1 (Figure 9a, lanes 2 and 4) or PC2 (Figure 9b, lanes 2 and 4) are not affected by the presence of overexpressed furin. When a similar experiment was performed in either COS-1 monkey kidney cells or in the rat insulinoma Rin m5F cells, an identical conclusion was reached, i.e. furin does not process either pro-PC1 or pro-PC2 (not shown). As a positive control, in these cell lines when either PC1 or furin was co-expressed with POMC, we observed the conversion of this precursor into β-lipotropin (Figure 9c), as previously reported [23,42]. Furthermore, the conversion of POMC by either furin or PC1 is specific to these convertases, as a similar experiment with PC2 results in the expected formation of β-endorphin (Figure 9c). Therefore the inability of furin to convert either pro-PC1 or pro-PC2 into PC1 or PC2 reflects the resistance of the prosegment of pro-PC1 and pro-PC2 to the intracellular cleavage by furin.

DISCUSSION

Figures 1 and 2 show that the initial cleavage of pro-PC1 into PC1 and of non-glycosylated pro-PC2 into non-glycosylated PC2 occurs as early as 1–3 min from the start of their synthesis. Consistent with the precursor–product relationship of pro-PC1 to PC1, we see a gradual increase in the 83 kDa form of PC1 over time (Figure 1a). As it would take about 1–3 min to complete the synthesis of one copy of a protein of the length of PC1 or PC2 [43,44], it is likely that, within the ER, the prosegment cleavage of pro-PC1 occurs co-translationally, or very soon afterwards.

Our results suggest that sulphation of PC1 and pro-PC2 occurs on a tyrosine residue. From the proposed rules of tyrosine sulphation [45], we predict that Tyr-694 [5] and Tyr-171 [8] could be the targetted residues in PC1 and PC2 respectively. As sulphation on tyrosine residues has been reported to occur within the *trans*-Golgi network [46], our data suggest that the processing of sulphated pro-PC2 occurs in this network.

In this work, we have demonstrated that the N-terminal propeptide cleavage of PC1 occurs in the ER and is more efficient than that of PC2. The processing of pro-PC2 is not favoured in the ER, resulting in longer retention in this organelle of the non-sulphated pro-PC2 than PC1. As efficient exit of proteins from the ER depends on their ability to fold correctly and to form higher-order structures, including oligomerization [44,47], the unequal rates of exit of PC1 and pro-PC2 from the ER may reflect the effect of the prosegment of PC2 on the rate of folding of this protein.

The inability of functionally active furin (Figure 9c) to excise the prosegments of PC1 and PC2 (Figures 9a and 9b) suggests that either this cleavage occurs autocatalytically, as described for pro-kexin [27,28] and pro-furin [29,31], or another unidentified proteinase is responsible for this processing. We therefore believe that, although furin can autocatalytically cleave its prosegment by an intramolecular mechanism [29,31], it is not capable of processing either pro-PC1 or pro-PC2. Recently [48], PC1 was shown to efficiently cleave *in vitro* the decapeptide [D-Tyr]-Arg-Ser-Lys-Arg↓Ser-Val-Gln-Lys-Asp encompassing the Lys-Arg-junction between pro-mPC1 and mPC1 [5], suggesting that PC1 has the capacity to undergo autocatalytic activation.

Proteins synthesized within the ER are transported to the Golgi complex and from there to their final destinations by a complex multistep dissociative transport mechanism [49]. The steps involved in this process have been elucidated by the use of specific drugs or treatments which disrupt transport at defined intracellular locations. For example, low temperature inhibits the transport of pancreatic secretory proteins [50]. Drugs such as CCCP deplete ATP sources and hence block the movement of proteins from the ER to the Golgi complex [38,51]. Brefeldin A affects early events in the transport of proteins by causing the disassembly of the Golgi complex and accumulation of secretory and *cis* and *medial* Golgi-resident proteins in the ER [39]. However, brefeldin A does not cause the relocation of proteins present within and beyond the *trans*-Golgi network [52]. Our data show that when GH_4C_1 cells were subjected to either one of these three treatments, the amount of PC1 (83 kDa) increased rather than decreased (Figure 3), suggesting that the excision of the prosegment occurs within the ER. However, we still do not know whether the processed PC1 is functionally active within the ER milieu. In experiments aimed at answering such a question for furin, it was shown that the activation of pro-von Willebrand factor [31] or of a mutant mouse pro-renin-2 [53] by furin is not possible in the ER.

The time at which a glycoprotein acquires resistance to digestion by Endo H is often used as a measure of the period required for a protein to travel from the ER to the *medial* Golgi [54]. The data in Figure 5(a) demonstrate that it takes less than 1 h for the appearance of the 84 kDa form of PC1 that is Endo H-resistant. In contrast, the intracellular pro-PC2 is still Endo H-sensitive after 6 h of chase and the secreted 68 kDa form is also sensitive to Endo H digestion (Figure 5b). These data highlight a major difference between the structure of the carbohydrate moieties attached to asparagine in PC1 and PC2, and further suggest that in PC2 they are of a high mannose type [54]. The importance of the glycosylation of PC1 and PC2 (as opposed to POMC) is emphasized by the degradation in ER of the non-glycosylated forms, obtained in the presence of tunicamycin (Figure 6). The nature of the ER resident enzymes responsible for this degradation are not known nor is the possible involvement in this process of heat-shock proteins such as BiP [55]. Our data further suggest that non-glycosylated PC1 and PC2 are more rapidly degraded than their proforms (Figure 6). Inhibition of asparagine glycosylation within the ER may either unfold or prevent oligomerization of these enzymes and hence expose them to the proteases that are responsible for clearing unwanted protein structures [55]. It would be informative to compare the relative importance of each one of the predicted three *N*-glycosylation sites in PC1 [5] and PC2 [8] in the protection of these convertases against degradation by ER hydrolases. In this context, it is interesting to note that *N*-glycosylation is important for the transit of pro-papain through the secretory pathway of insect Sf9 cells and is a prerequisite for the production and secretion of functional pro-papain [56].

The major secreted form of PC1 (84 kDa) from the GH_4C_1 cells is enzymically active on a number of peptide substrates *in vitro* [48]. The transformation of the 83–84 kDa form of PC1 into the 66 kDa form occurs rather slowly [36] and the extent to which this occurs is cell type-dependent, e.g. in AtT-20 [36] versus GH_4C_1 cells (this work). We still do not know whether the 66 kDa form of PC1 is active. Furthermore, in view of its late appearance, its intracellular function is not yet clear. We also note that unlike for PC1, no report showed the presence of a C-terminally truncated form of PC2 in any tissue studied [36,57]. In contrast, immunoblots of rat adrenal medulla also showed the presence of an N-terminally truncated form of PC2 which migrated with an apparent molecular mass of 40 kDa [57], and which presumably would be inactive as part of the catalytic segment would be missing [7,8].

In conclusion, it will take a concerted effort using a battery of antisera specific for various PC forms, various secretagogues, biochemical and electron microscopic analysis, before we can define in more detail the molecular sizes of the enzymes found in each organelle. Further work is necessary to define the various post-translational modifications of the processing enzymes and their effect on the activity and sorting of these convertases. It is hoped that the generation of these data and those obtained from comparative enzymic activity studies of the various forms of PC1, PC2 and furin will lead to a better understanding of the complex process of proprotein and prohormone activation.

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